ANALYSIS OF QUALITY AND TECHNIQUES FOR THE HYBRIDIZATION OF *CORDYCEPS SINENSIS*

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ABSTRACT:

In the course of our research with cultivated *Cordyceps sinensis*, we have noted a greater diversity of compounds from different strains of this single species, than in almost any other organism we have analyzed. Due to this great difference in chemical composition, a wide range of quality is found in cultivated *Cordyceps*. This article details the unique methods used to develop hybridized strains of *Cordyceps* utilizing rattlesnake venom to trigger somatic fusion of dissimilar mycelial strains. This results in the reproducible production of cultivated *Cordyceps* containing quantities of recognized bioactive compounds equal to or greater than the quantities found in wild collected *Cordyceps*. These techniques and methods offer great promise in allowing cultivators of *Cordyceps sinensis* and other *Cordyceps* species to take their artificially cultivated products to a higher and more consistent level of quality, and provide protocols for analysis of *Cordyceps* quality using HPLC and GC techniques.

KEYWORDS:

Cordyceps sinensis, Snake venom hybridization, HEAA, Cordycepin, adenosine, Hydroxyethyladenosine, deoxynucleosides, Cordyceps culture parameters, *Cordyceps* analysis.

INTRODUCTION

Cordyceps sinensis is an insect colonizing fungus of the ascomycetes family, found at high altitudes on the Qinghai-Tibetan plateau. This fungus lives primarily in the larvae of one particular species of moth, *Hepialus armoricanus*. It is occasionally found growing on other moth species as well. The normal range of this fungus is above 2000 meters elevation, and it has been found as high as 6000 meters. There are also many other species of the genus *Cordyceps*, which all seem to have potent biologically active compounds present. The genus has been shown to produce some potent antibiotics, immune stimulants, antiviral and antitumor agents. (Zhou, Halpern and Jones, 1998 [1]; Hobbs, 1986)

Cordyceps sinensis, which is known to the Chinese as “Dong Chong Xia Cao” and to the Japanese as “Tochukaso” has been used in medicine for a very long time. The first known written record on the medicinal uses of this herbal medicine was in the Ben-Cao-Cong-Xin (*New Compilation of Materia Medica*) by the author Wu-Yiluo. Written around the year 1757 AD, this early medical text lists the traditional usage of *Cordyceps* as going to the Lung and Kidney meridian and being useful as a “Lung Protectorate”, for “Kidney Improvement” and as a “Yin/Yang double invigorant”. *Cordyceps* in Traditional Chinese Medicine (TCM) was, and usually still is, prepared by cooking the whole caterpillar/fruitbody combination in chicken or duck soup. It has been used this way for the treatment of many conditions, such as respiratory diseases, renal dysfunction, hyperlipidemia and hyperglycemia. (Zhou, Halpern and Jones, 1998 [2])

This would seem to be a lot of medicinal claims for a single substance. But the observations of many generations of medical practitioners has validated and confirmed this belief. People tend to be very good observers of nature. Especially when the observations are proven over a long period of time, as has been the case with *Cordyceps*, there is sufficient background for further research into this herbal substance for use in modern medicine.

Since the 1950's attempts have been made to cultivate *Cordyceps sinensis* and other *Cordyceps* species, and many of these artificially cultivated *Cordyceps* have been available in the international marketplace since the 1980's. Due in part to this wide availability of *Cordyceps*, the worldwide demand for this previously rare health supplement has greatly increased in the last 20 years. This has led to over-harvesting of the wild stocks and a subsequent shortage of the natural *Cordyceps*. With the increasing scarcity of *Cordyceps* in the wild (Chen S, Yin D, Li L, Zha X, Shuen J, Zhama C., 2000) the prices for this popular health supplement are ever increasing, which has lead to more and more companies producing artificially cultivated *Cordyceps* for the nutraceutical and pharmaceutical markets. This increase in demand and supply has lead to a wide variation in the purity and quality of the *Cordyceps* products available. It has also lead to a large number of counterfeit and adulterated products being sold under the name "*Cordyceps*".

There have not been any widely accepted standards available on which to base the analysis of *Cordyceps* for quality purposes. Some manufacturers analyze for Adenosine, while others analyze for Cordycepin, while yet others analyze for polysaccharides or other compounds. This has lead to great confusion in the publics' mind as to which *Cordyceps* is best for them to buy. In

the course of our research into this quality issue, the first thing we had to do was to develop analytical protocols and choose prototypical compounds to test for, in order to establish a 'Quality Index' by which different strains and commercial products could be graded. These protocols and compound descriptions are found in the section titled METHODS.

There is debate among many scientists at present whether the species of the genus *Cordyceps* are in fact single organisms or if they are symbiotic colonies of more than one organism. Perhaps what we are calling *Cordyceps sinensis* today, will one day be known as a fungal/bacterial symbiosis. DNA sequencing has proven inconclusive in this regard as the DNA sequence tends to change with time, even as if the fungus were incorporating some of the insect DNA into its own DNA code for the initiation of its fruitbody form, then losing the insect DNA when it goes back into its mycelial form. Microscopic examination of growing *Cordyceps* mycelium often reveals some very interesting morphology, including the concurrent anamorphs of filamentous mycelium and rapidly moving single celled yeast-like morphological forms. This has been seen in other *Cordyceps* species as well, such as *Cordyceps sobolifera*. Many times we have observed what appears to be inner-cellular symbionts, a small, spherical, rapidly moving, apparently single celled organism living within the long, bamboo-like hyphal strands. When we observe this growth form, these secondary innercellular objects occur in about 70-80% of the mycelial cells observed, with anywhere from one to five individually moving spheres per mycelial cell. These are easily observed in the living culture under 1000-power magnification with normal back lighting.

MATERIALS

MODERN CULTIVATION OF *CORDYCEPS SINENSIS*:

Since native *Cordyceps* (wild *Cordyceps sinensis*) is quite rare and very expensive, there has been a lot of research into methods for cultivation of this fungus. The strain presently known as CS-4 was one of the first commercial strains of *Cordyceps* isolated and brought into cultivation. Known by the Latin name of *Paecilomyces hepiali* Chen, the aseptically fermented mycelium of this strain underwent extensive human testing and clinical trials during the 1980's and resulted in a commercial product with wide usage in China, known as Jin-Shui-Bao capsules. More than 2000 patients were involved in the clinical trials with CS-4 during the pharmaceuticalization of Jin-Shui-Bao, and the chemical composition, therapeutic activity and toxicity are very well known for this strain. (Bau, 1995)

A number of other strains have been isolated from wild *Cordyceps* since then that are presently under commercial cultivation around the world. These strains are each so different from the original starting *Cordyceps* and from each other that they have been given many different genus and species names. Even though the parent fungus is the same in each case, the resultant asexual mycelial growth forms are characteristically different enough in morphology and chemistry that they are considered different species by many taxonomists. Table 1 lists some of the different strains isolated from wild *Cordyceps sinensis*. (Yin and Tang 1995; Zhao, Wang, Chen, Li and Qu, 1999)

Table 1

Latin binomial	**Isolated by**	**Commercial product**
Cephalosporium sinensis	QingHai Institute of Livestock and Vetrinary sciences	NingXinBao
Paecilomyces sinensis Cn80-2	FuJianQingLiu County Hospital	various
Scydalilum sp.	Sanming Mycological Institute	SM1H8819
Scydalilum sp.	Chinese Navy Institute of Medicine	832
Hirsutella sinensis		various
Mortierella hepiali Chen Lu		various
Topycladium sinensis		various
Scytalidium hepiali G.L.Li		various

ADULTERATED AND COUNTERFEIT *CORDYCEPS SINENSIS*:

With the opening of China to business with the West during the 1970's, many people in countries far from China were exposed to the benefits found in Traditional Chinese Medicine. Along with this exposure to the traditional medical methods came a great demand for the various herbal compounds used in that medical system. This great worldwide demand for *Cordyceps*, and the enormous cost of the wild collected variety has led to many unscrupulous manufacturers and distributors providing adulterated and counterfeit *Cordyceps* in the world market. (Hsu, Shiao, Hsiea and Chang 2002) Most of the Western world prefers their medicine to come in clean white bottles and neat little capsules, rather than in the whole caterpillar form. This makes it even easier and more tempting for some suppliers to sell just about anything under the label of

"*Cordyceps*". Most consumers of natural *Cordyceps* already know that it is normal practice for collectors to insert small segments of twigs or even pieces of wire into the body of the caterpillars to increase the weight. (Wu TN, Yang KC, et. al., 1996) And most consumers of capsulated *Cordyceps* in the western countries do not even know what real *Cordyceps* tastes or smells like. We have analyzed some specimens of "*Cordyceps*" capsules which contained nothing but rice flour, and other samples which contained nothing but nutmeg.

In an attempt to establish a reliable method for identifying "real" *Cordyceps*, we started analyzing all of the available cultivated *Cordyceps* products, both consumer packaged products and bulk raw material products, grown by nearly all of the commercial cultivators and suppliers worldwide. What we found was shocking. Nearly all of the commercially available *Cordyceps* products available in the United States (nearly all of which were imported from China) contained no detectable amounts of *Cordyceps* whatsoever. The results of testing on American produced *Cordyceps* were a little better. With American *Cordyceps*, in every case we were able to recognize the characteristic analytical signature of *Cordyceps*, (refer to plot 1 to see the characteristic analytical signature used in proofing specimens as being *Cordyceps* or not), but in none of the American samples was there any significant amount of active ingredients. The American grown *Cordyceps* products consisted almost entirely of unconverted grain substrate upon which the *Cordyceps* is grown.

METHODS

TEST METHODS AND STANDARDS OF QUALITY:

The methods for analyzing *Cordyceps* quality have not yet become standardized throughout the world. Every lab that is conducting this type of testing uses their own methods and their own standards. So when we first began our analysis of *Cordyceps* in 1999, we had to develop our own test methods. We tried many different test protocols before settling on the following two, as being accurate, repeatable and relatively economical.

GAS CHROMATOGRAPHY WITH MASS SPEC DETECTION (GC/MS):

Trimethylsilyl Derivative Method: Starting with well dried and finely ground powder of the raw test sample, 20 mg is added to 0.3 ml of derivatizing agent (BSTFA) and 0.3 ml of acetonitrile. This mixture is heated for 20 minutes at 60 degrees C, which yields a Trimethylsilyl derivative, which carries the material through the GC for detection with a mass spectrometry detector. This test method is simple and quick, and it yields a positive/negative answer as to whether the test sample is actually *Cordyceps* or not. This method can also be used for quantification of the target compounds, although the next method is more accurate and more suitable for complete target compound quantification:

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY / MASS SPEC DETECTION:

Powdered samples (2.0g) were defatted by decanting with hexane (3 x 50ml) and dried in vaccuo. Samples were dissolved in 0.1M TBE (Tris-borate-EDTA) buffer (pH 8.5 with 0.1N NH_4OH) (100ml) and sonicated for 30 minutes at 40°C. An aliquot (10ml) of the sample was then passed through a C-18 Sep-Pak that had previously been pre equilibrated with 0.1M TBE (Tris-borate-EDTA) buffer (pH 8.5 with 0.1N NH_4OH). The eluent was collected, and the Sep-Pak further washed with the equilibration buffer to give a final eluent volume of 20 ml. After

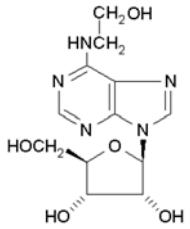
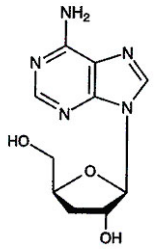
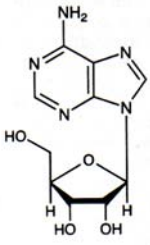
thoroughly mixing the sample was filtered through a 0.45 micron PVDF membrane and placed into suitable vials for HPLC-MS analysis. The chromatography was performed on a Waters 2695 separation module using a Wako Wakosil-II 5C18 HG column (5 mum, 15 cm * 4.6 mm i.d.) at 45°C with gradient elution of H_2O:methanol (1 ml/min) from 22:3 to 77:23 in 19 min, then to 18:7 at 24 min and 27:23 at 39 min. The chromatographic eluent was passed into a Vestec particle-beam interface for solvent removal and particle atomization and then via Teflon transfer line into the mass spectrometer using a helium carrier gas. Detection was performed on a Finnigan TSQ7000 triple-quadrapole mass-spectrometer in positive ion mode with full scan centroid data collection (50-1000 m/z). MS-MS experiments using an argon collision gas were used to verify the identity of unusual nucleotides for which no primary standards were available.

STANDARDS OF QUALITY or 'QUALITY INDEX'

Quality determination in *Cordyceps* for the Health Supplement industry has been haphazard up until now, as there have been no universally recognized test methods for analyzing this particular supplement. Each company producing or supplying *Cordyceps* has used different tests, or tested for different substances in order to show that their product stands out above all the rest. Some analyze for Adenosine, some for Cordycepin, some for Cordycepic Acid, and some for particular sugars or total polysaccharides. While all of these tests have some usefulness in determining relative quality, none of these tests by themselves are in any way meaningful when it comes to whether the product in question will yield good results when used as medicine for humans. Almost all of the samples of wild collected *Cordyceps* analyzed very similarly in chemical composition, but we quickly realized that there was a tremendous variation in the secondary metabolite compounds present in cultivated *Cordyceps sinensis* and other *Cordyceps* species. A

greater variation in compounds than we had found in any other organism. So our first step was to determine which compounds we could use as targets to equate with medicinal potency. After a thorough review of the literature, the nucleosides, and specifically the deoxy-nucleosides were determined to be the most reliable indicators of potency. This class of compounds has been carefully studied and reported in the scientific literature, and was the class of compounds showing the most variation in different samples of cultivated *Cordyceps*. Furthermore, many of the deoxy-nucleosides are found in no other organism, or at best, a very limited number. We chose the compound N^6-(2-hydroxyethyl)-Adenosine as our indicator compound, since we found this in all specimens of *Cordyceps* tested, but have not found it in any other organism. This compound, along with Adenosine and 3'deoxyadenosine (Cordycepin) were used in summation as the quality indicator for comparison of different strains and production methods of *Cordyceps*. In other words, the quantities of the three compounds (in percentage w/w) were added together to come up with a numerical Quality Index for *Cordyceps*. (Furuya, Hirotani and Matsuzawa, 1983) Structures for these three compounds are shown in Table 2:

Table 2 – HEAA STRUCTURES

		
N^6-(2-hydroxyethyl)-Adenosine	Cordycepin (3'deoxyadenosine)	Adenosine

There are a number of other interesting deoxy-nucleosides produced by *Cordyceps sinensis*, such as the compound 2',3' deoxyadenosine, which is marketed in the United States as a drug for the treatment of AIDS under the trade name "Didanosine". There are also several varieties of deoxy-uridines present, but there is not much literature on these compounds yet, so we decided not to use these as quality index markers. In time we will learn what effect these unique compounds have in the human body and may find that these compounds are as important a group as the adenosine class of compounds, which we refer to in this paper as **HEAA** (**H**ydroxy **E**thyl **A**denosine **A**nalogs).

We find that the HEAA content is a much more reliable indicator of *Cordyceps* quality than could be determined through testing of the polysaccharides. The reason for this has to do with the methods of analysis for polysaccharides, which is usually done by wet-chemistry methods; breaking the polysaccharide bonds through acid hydrolysis or enzymatic activity and then measuring the quantity of simple sugars present after cleavage. We find this method unreliable as a quality indicator, since the residual sugars tell nothing of their source or their linkage characteristics (ie: alpha-bonded or beta-bonded), and the test results are easily altered through the addition of starch or other polysaccharides to the raw material at the manufacturing stage. Furthermore the resultant simple sugars are not unique to the *Cordyceps* in question. Rather, by using the HPLC/MS or GC/MS method and testing for relatively rare nucleosides, any sample of *Cordyceps* from any source is quickly and easily analyzed and a numerical quantity index can be applied to it. When this method becomes universally applied in the health supplement industry, producers will be forced to address the quality issue of their products, and the industry as a

whole will benefit. There will be much less adulterated or counterfeit product placed on the market and consumer confidence will grow as a result. It is hoped that by the application of the test methods given here, such deceptive production practices will stop.

CULTURE METHODS AND SUBSTRATES:

In looking at the variations in quality from different strains and producers of *Cordyceps*, one must wonder what is it that causes this to be so. After all, a tomato is a tomato, no matter where it is grown. Yet with *Cordyceps*, even the same strain (CS-4) grown by different cultivators turns out to be entirely different from a standpoint of active ingredients.

In looking into this question, it is first important to realize that there are two different methods used today in the cultivation of *Cordyceps*. There is the method primarily used in China, known as Liquid Culture or Fermentation, in which the organism is introduced into a tank of sterilized liquid medium, which has been formulated to provide all of the necessary nutritional components for rapid growth of the mycelium. After growth in the liquid medium, the mycelium is harvested by straining it out of the liquid broth and dried, after which it can be used as-is or further processed. Generally in this method the extra-cellular compounds, which were exuded by the fungus during the growth cycle, are discarded with the spent broth. This represents a major loss of bioactive compounds as many of the active ingredients are extra-cellular in nature, and are found only in small concentrations in the mycelium.

The second cultivation method is the solid-substrate method followed by most growers in Japan and America. In this cultivation system the mycelium is grown in plastic bags or glass jars full of

sterilized medium, which is almost always some type of cereal grain. This grain is usually rice, wheat or rye although many different types of grain have been used. After some period of growth, the mycelium is harvested along with the residual grain. While this is an easily mastered and low capital-investment cultivation technique, the negative side of this method is that the grain content is usually greater than the mycelium content. In many cases the solid-substrate grown mycelium we tested contained greater than 80% residual grain. However, a bonus to this method is that the extra-cellular compounds are harvested along with the substrate and mycelium.

Cordycepin is an example of one of the compounds that are primarily extra-cellular in nature. Many tests have been done on cultured *Cordyceps* mycelium for the presence of Cordycepin and these show that Cordycepin is generally present in solid-substrate grown *Cordyceps*, and usually it is not present in liquid-cultured *Cordyceps*. The presence or absence of Cordycepin is dependent upon, among other factors, by which method the mycelium was grown and harvested.

We can see from this that the culture method itself has an effect on the quality of the resultant *Cordyceps* product. Beyond the methodology itself, the next most important factor in the production of particular secondary metabolites (or target medicinal compounds) is the nature and composition of the substrate itself. (Zhang, Zhao, Wu, Bai 1992) While it would seem that a substrate that favors rapid and strong growth of the mycelium would be an ideal substrate to use, but this is not necessarily the case. Substrates are chosen on availability and price, or on historical usage or preference in handling. But rarely have they been chosen on the basis of the end compounds produced. In fact, the only way to determine whether the substrate being used is

the best choice or not, is to compare the resultant product after growth on that particular substrate with some standard. If the end goal of production is Cordycepin or Didioxyadenosine (or some other specific compound) - as it is with some of the pharmaceutical companies - then the analysis is fairly straightforward. Just look for the amount of Cordycepin or Didioxyadenosine present and adjust substrate composition accordingly. However business in the health supplement industry is rarely so simple. First we have to assume that we know what it is that we are looking for. Since natural products such as *Cordyceps* are chemically very complex, the truth is that we do not really know all of the components that are bioactively important.

With this realization in mind, we set out on a mission: To produce the best *Cordyceps* possible. What is the best? Since we did not know the answer to that question, we decided to try to copy the natural, wild collected *Cordyceps as closely as possible*. We attempted this by altering the substrate composition and analyzing the resultant mycelial product for known bioactive compounds. Then altering the substrate again…and again…and again. We did this through several hundred different substrates and through many thousands of kilograms of resultant product. What we found was that there was not any single method, strain or substrate we could use that would yield the results desired.

SUBSTRATE:

The substrate of choice for most Chinese growers is a liquid media based upon silkworm residue, with added sugar, carbohydrates and minerals. This seems a logical choice, since this mushroom is found in nature growing on insects. Dried silkworm bodies are the by-product of an existing industry and have little other use. They are therefore readily available and cheap. This silkworm-

based substrate seems to yield a relatively high quality product. The only problem with silkworm- residue based substrate is that in the United States, the FDA requirements are for mycelial products to be produced on a normally consumed human food source. Silkworms do not fit into that category. They are also not available as a raw material source to most of the worlds *Cordyceps* cultivators.

The most usual substrate for Japanese and American growers is rice. It was determined in our trials that rice is not a suitable substrate for *Cordyceps* production if the target medicinal compounds are considered. Rice does not allow the full range of secondary metabolites to be expressed by the fungus, and rice grown *Cordyceps* has tested inferior in all of our analyses of active ingredient. There is rarely any appreciable amount of Adenosine or Cordycepin present in rice-grown *Cordyceps*. Furthermore, there are growth-stunting metabolites which build up in the substrate when *Cordyceps* is grown on rice, limiting the growth stage to only about 22-24 days, and allowing no more than about 40% of the rice to be converted into mycelial mass. This figure of 40% represents the high end of conversion, and is usually around 25-30%. This means that when *Cordyceps* is grown on rice then dried and powdered, the resultant product is actually between 60-75% unconverted rice flour.

Rye grain is another substrate often used for solid culture, and it yields a higher quality product than rice, as long as some vegetable oil as an amendment is added to the growth medium at the time of substrate makeup. The oil provides necessary nutrients, which the organism utilizes for bioactive compound production. Rye has other disadvantages though. The compounds in rye, which give it that characteristic rye smell and taste, are not broken down by the *Cordyceps* and

concentrate in the final product. This rye taste and smell overcomes the characteristic *Cordyceps* taste and smell, and even though the resultant product is of better quality than the rice grown mycelium, (as determined through analysis) there are certain perceptual problems that needs to be overcome by the buyer to make this an economical substrate alternative. Rice-grown *Cordyceps* may seem like a better product to the average buyer because the rice does not mask the characteristic *Cordyceps* smell and taste. Most buyers in the health supplements industry tend to purchase bulk products on perception and faith rather than requiring an independent analysis. Rye also has growth-limiting factors, which causes the *Cordyceps* growth to stunt at about 28-30 days, although this can be overcome to a slight degree with the addition of about 1% ground oyster shell buffer to the medium at time of make-up. We tried many other sources of calcium, but none seemed to work as well as the oyster shell calcium.

Millet is a very good choice of substrate when it is available. It has no strong taste or smell of it's own, it does not stunt the growth to any significant degree and it allows for the full expression of the secondary metabolites by the organism. It has another problem though, which is the high ratio of chitinous outer husk layer to starch. This outer husk is not broken down and represents a large portion of the final product weight, about 15%. The chitinous husk cannot be removed from the grain ahead of time, since doing so causes the grain to become too sticky during sterilization and a high degree of anaerobic contamination follows. The husk can be removed from the final product through mechanical means such as a Time-Of-Flight separation process, or the product can be used for hot water extractions or other processing. *Cordyceps* does not grow as fast on millet as it does on other grains, but the end product quality is higher.

White milo grain, also known as white kaffir corn or white sorghum is an excellent choice of substrate. The red variety of milo does not work nearly as well as the white variety as a substrate. White milo has all of the best characteristics; it is cheap, it has a high starch to husk ratio, it does not stunt the growth, it allows the full expression of bioactive compounds to be expressed and has no strong odor or taste of its own to compete with the taste and smell of the resultant *Cordyceps* product. White milo when used alone however lacks some essential ingredients required for optimum growth by the *Cordyceps*. The addition of some portion of millet to the white milo speeds up the growth by a factor of 6 times. The millet to milo ratio is optimum at 1 part millet to 4 parts white milo.

Many farmers grow both white milo and the red milo in the same fields, or store them in the same silos, or otherwise do not keep the white and red separated. This is to be avoided when used as a *Cordyceps* substrate, since a small proportion of the red mixed in with the white can drastically reduce the growth rate and overall quality of the final product.

So from our substrate testing it was determined that the ideal medium for solid substrate growth of *Cordyceps* is as follows: 1 part white proso millet (husk on) to 4 parts of white milo (husk on), with the addition of 0.8% w/w of ground oyster shell and 1% w/w vegetable oil (peanut oil or soybean oil). Add water to equal 50% total moisture in the sterilized substrate. Precooking the grain mixture for 4-6 hours prior to sterilization tends to trigger a much faster growth response from the *Cordyceps*. On this medium, *Cordyceps* can be grown for long periods of time, allowing nearly complete conversion of the substrate to mycelium (96%+) and the full expression of secondary metabolites from the *Cordyceps*. The resultant *Cordyceps* when grown

on this substrate is about 3-4% residual grain, or about 96-97% pure mycelium. The real benefit to this method of growing is the capture of the entire compliment of extra-cellular metabolites produced throughout the entire growth process. With the addition of certain growth triggering compounds to this mixture, *Cordyceps sinensis* is easily induced to fruit in culture without any insect material being present. However the formation of the fruitbody on this medium does not result in any significant change to the analytical chemistry profile.

TABLE 3 – COMPARISON OF DIFFERENT SUBSTRATE COMPOSITIONS ON CORDYCEPS QUALITY AS DETAILED IN TEXT

SUBSTRATE	SPEED OF GROWTH	% RESIDUAL SUBSTRATE IN FINISHED PRODUCT	SUBSTRATE QUALITY INDEX [1]
Silkworm / Sugar (Chinese)	Fast	15-20%	Not FDA allowable [2]
Millet	Medium-Fast	15%	90
White Milo	Medium	2-5%	90
Millet & White Milo 1:4 ratio	Fast	2-5%	100
Rye	Medium-Slow	40-50%	60
Oats	Medium	20-30%	80
Wheat	Medium-Slow	50-80%	60
Brown Rice	Medium-Fast	60-80%	60
Soy Beans	No to Slow	90-99%	Not a good substrate [3]

Notes: 1) FDA Regulations cpg 585.525

2) This 'substrate quality index' is based upon the analytical profile of *Cordyceps sinensis* grown on each substrate, and to a lesser degree it is subjective and is based

upon the authors' experience of the growth characteristics on each, such as speed of growth, tendency to contaminate, ease of sterilization and ease of handling. The best substrate found was the Millet/Milo mix, which was assigned a Quality Index value of 100. All the other substrates are judged in relationship to this.

3) Soy Beans are not an acceptable substrate in these Authors experience. We tried to grow a number of different strains of *Cordyceps sinensis, Cordyceps militaris, Cordyceps ophioglosoides and Cordyceps sobolifera* on different soy based compositions, but had virtually no success. We are aware that there are commercial products on the market that advertise being grown on soy, however we can only guess what tricks those cultivators have developed to insure good growth on this medium. We mention soy-based substrates here only as a guide for the beginning cultivator, so they do not waste too much time trying to follow a route that we have found unacceptable.

CULTURE PARAMETER MODIFICATION: LOW TEMPERATURE HYPOXIA

Using the above-described substrate, the complete chemical profile of the cultivated C*ordyceps* still will not approach that of the wild collected *Cordyceps* unless it is grown under very specific conditions. *Cordyceps* sinensis produces a relatively large amount of free Adenosine when grown at normal atmospheric oxygen levels and room temperatures. It will also produce a large quantity of Uridine and Guanidine. But there is very little if any Cordycepin produced, and virtually no Hydroxyethyl Adenosine. For the organism to produce these compounds, it needs to be growth-stressed through the absence of oxygen, a drop in temperature and the total absence of

light. Just growing it under cold and anaerobic conditions from the start will not do the trick, since when *Cordyceps* is grown under those conditions it forms a yeast-like anamorph that has a very different chemical profile. It must first be grown hot and fast, then tricked into converting its 'summertime' metabolites into the target medicinal compounds we are looking for. To get these target compounds, we found that we needed to follow a strict growth protocol: After inoculation on to the millet/milo substrate, the *Cordyceps* is grown at 20-22 degrees C, in diffuse light and at sea level atmospheric oxygen for 28-30 days. It is then moved into a specially controlled environmental chamber, where the oxygen content is dropped to 50% atmospheric. The remainder of the growth atmosphere is made up of Nitrogen, Carbon monoxide and Carbon dioxide. The temperature is dropped to 3 degrees C, and all light is excluded. It is held under these conditions for about 15-20 weeks. This results in much of the Adenosine being converted to Cordycepin, Dideoxy-adenosine and Hydroxyethyl-adenosine. Many other unique nucleosides are also produced, **with a final chemical profile identically matching that of the wild *Cordyceps*** as can be clearly seen in Plot 1.

HYBRIDIZATION:

Once we had developed the substrate and growth parameters to optimize the target compounds, we started looking into the chemical profile differences from different strains of *Cordyceps sinensis*. Since there were so many strains of *Cordyceps*, and each strain has its own unique chemical profile, we tested all of the strains we were able to obtain. None of the known strains was shown to produce nearly the quantities of active ingredients found in the wild *Cordyceps*. So we started experimenting with ways to quantitatively increase the target compound production through the hybridization of *Cordyceps* strains; to cross breed them in order to gain greater

production of target compounds. This was quite a challenge. Since spore collection and separation is very time consuming and results in entirely too many unknown variations, we felt this method would take too much time before we had reliable results. Rather we took a novel approach. We experimented with various ways to get different strains of the fungi to perform their own nuclear fusion. There are several chemicals known to trigger this exchange of genetic material between unlike cells. Nicotinic acid for instance, can be used to create hybridized mycelium. This compound is difficult to use and yields unreliable results. After trying several different compounds to trigger this fusion, what we settled on was snake venom.

SNAKE VENOM AS A HYBRIDIZATION AGENT:

We used purified snake venom from the Western Diamondback Rattlesnake (*Crotalus atrox* see illustration 2) [Sigma Scientific, St Louis Missouri, USA] for our hybridization techniques. The snake venom is added to the agar medium in quantities that alters the growth but does not prove toxic to the strain in question. This range of snake venom is from 10 mg to 30 mg per 300 ml of agar medium. The venom is not heat stable and must be added aseptically after sterilization of the medium. The agar used for this hybridization is an Aloha Medicinals Inc. proprietary agar named R7 Agar, consisting of malt extract, activated carbon, minerals and humus – the carbon-rich ash residue from a coal burning industrial process. For the exact recipe see table 4. Other agars could probably be used as well. This just happens to be our production agar that we use everyday, and once we found that it also worked with the snake venom for hybridization, we found no reason to experiment with any other agar.

TABLE 4 – SNAKE VENOM / R7 AGAR RECIPE

2.1 L	Distilled Water
50 g	Light Malt Extract
34 g	Agar
10 g	Humus
5 g	Activated carbon
1 g	$MgSO_4$
10 ml	1% KOH solution
As Required	*C. atrox* venom

HYBRIDIZATION TECHNIQUE:

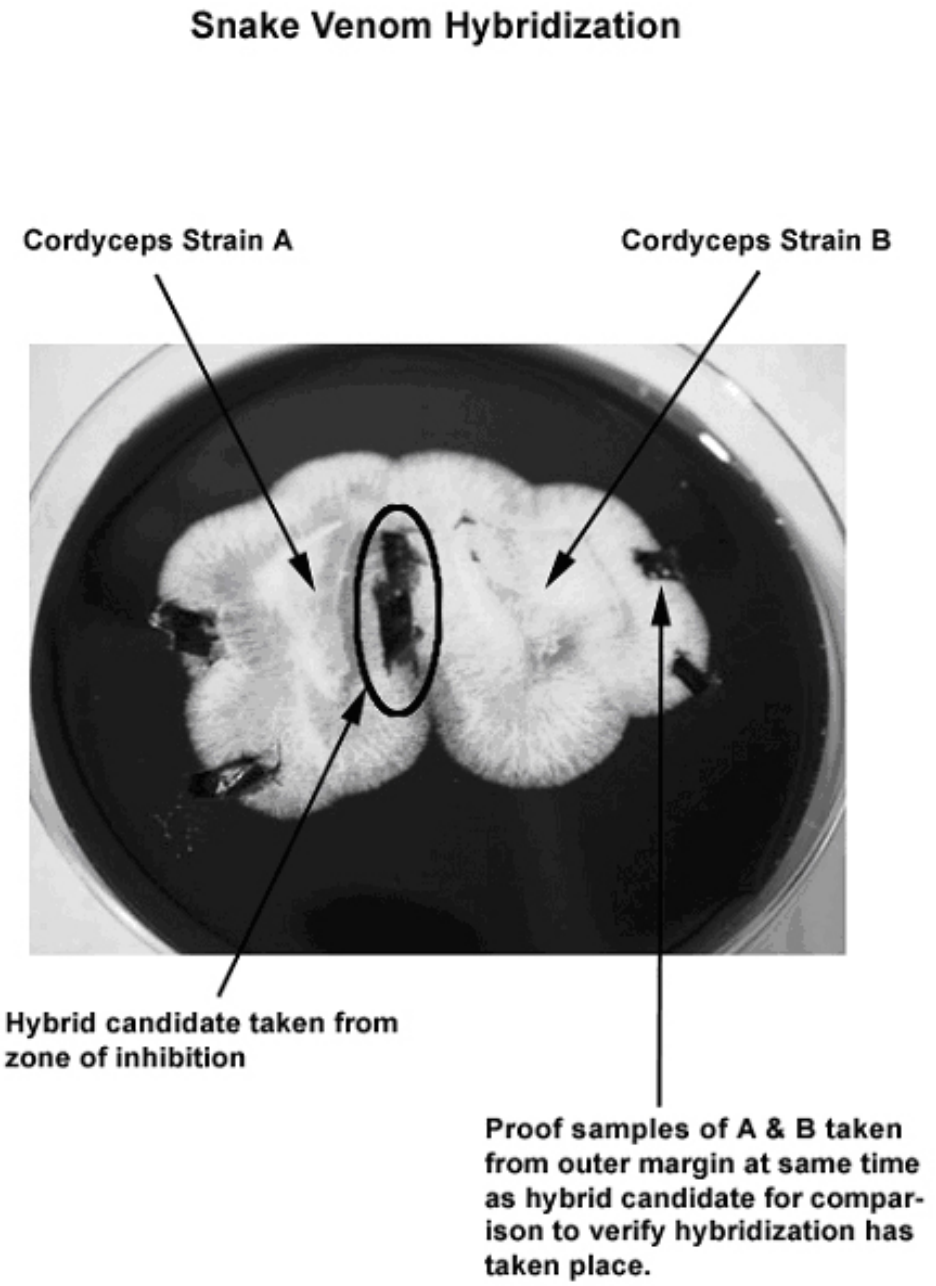
Petri dishes of this R7 agar medium are inoculated with mycelium from two different strains of the *Cordyceps* genus. These are usually two varieties of *C. sinensis*, although we have also crossbred *C. sinensis* with other *Cordyceps* species such as *C. militaris, C. sobolifera* and *C. ophioglosoides.* These different strains when inoculated together onto one petri dish will normally grow towards each other until they almost meet, at which point they form a zone of inhibition, where neither strain can grow. Eventually, one strain may prove stronger than the other and overgrow the plate, but they will remain genetically distinct: two different cultures residing in the same petri dish.

With the addition of a sufficient quantity of snake venom to the agar, we found that what happens is the two cultures grow towards each other until they meet and form their mutual zone

of inhibition. This period of inhibition is short lived however, for in only about 2 or 3 hours the colonies each start sending out mycelial strands into this no-mans land, the zone of inhibition. These strands grow together and exchange nuclear material through their venom-weakened cell walls. They form a hybrid strain at this point of mutual contact. A new strain, one that is distinctly different from either of the parent strains. Within about 4 hours after first forming the zone of inhibition, the hybridization is complete and the colonies resume rapid growth towards each other. They become three colonies rather than the original two. There then exist in the same plate the original two colonies and a genetically distinct third…The Hybrid.

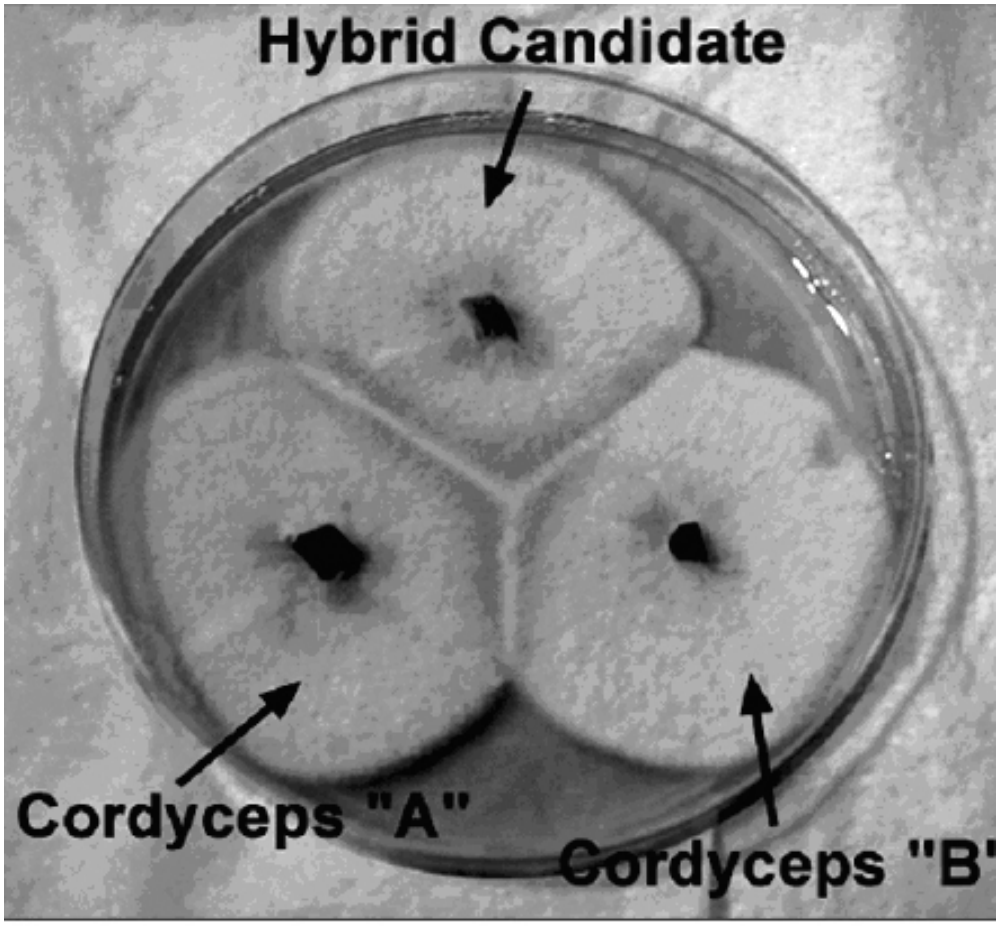
A section of the newly formed hybrid is carefully removed from the original zone of inhibition at the precise time that the colonies begin to fuse. That is during hour 3-4 after the initial meeting of the colonies. The hybrid is transferred to a new petri dish containing normal (non-snake venom) agar. Our quick method of determining hybridization is to inoculate a new dish containing normal agar with tissue samples from all three strains, the original two and the suspected hybrid. If hybridization has failed to occur, then the suspected hybrid will readily fuse with either one or the other of the original colonies, proving that our suspected hybrid is not genetically distinct from the original, and we start again. But if the hybridization has in fact taken place, these are now three distinct colonies, and will form a mutual three-way zone of inhibition. These can be clearly seen in Illustration 2 where there are distinct zones of inhibition shown between three different colonies in the plate. See Illustration 1 and illustration 2

Illustration 1


Snake Venom Hybridization
Cordyceps Strain A
Cordyceps Strain B
Hybrid candidate taken from zone of inhibition
Proof samples of A & B taken from outer margin at same time as hybrid candidate for comparison to verify hybridization has taken place.

NOTE:
Agar shown in this illustration is proprietary R7 agar with .0001% *Crotalus atrox* venom added. Cultures in this photo are *Cordyceps sinensis* CS_4 and *Cordyceps sinensis* SC1007 strains.

Illustration 2



Once a hybrid is confirmed, it is tested for growth parameters. If it appears to be a vigorous and hardy grower on our substrate of choice, we grow out a quantity of mycelium, harvest it and analyze it for active ingredients. Through repeated testing in this way we were able to create the hybrid strain shown in Plot 1; a hybrid strain that is easily grown in solid substrate culture, with a potency greater than any other cultivated strain and at least equal in potency to the highest

quality wild *Cordyceps*. We are referring to this new strain as *Cordyceps sinensis* Alohaensis. We are presently continuing this hybridization work with other species of *Cordyceps*, for the production of very specific target compounds. Top quality *Cordyceps* is no longer a health supplement only for the very rich. By utilizing these new methods of cultivation, the best quality *Cordyceps* is now within economic reach of even the common man throughout the world.

RESULTS:

ANALYTICAL RESULTS:

We conducted analysis over the course of 4 years on samples from what we believe to be every commercial producer of cultivated *Cordyceps* in the world. The results obtained through testing these approximately 100 samples from producers in 4 countries is shown in table 3:

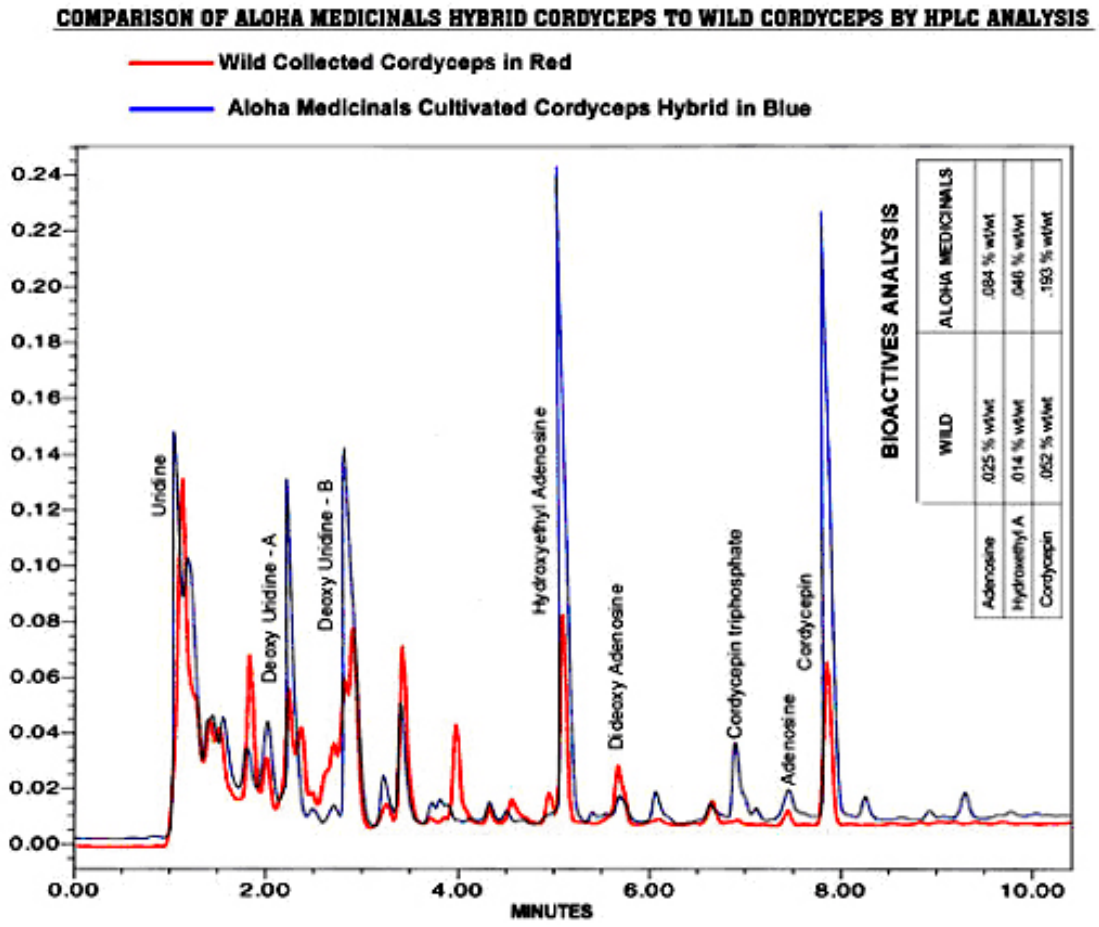
Table 3 – QUALITY INDEX OF CULTIVATED CORDYCEPS SINENSIS

	American mycelium	Oriental mycelium	Standardized Extracts	Wild *Cordyceps*	Aloha Medicinals hybrid *Cordyceps*
HEAA Range	0.00%-0.041%	0.00%-0.071%	0.050%-0.073%	0.071%-0.100%	0.190%-0.323%
HEAA Average	0.005%	0.025%	0.070%	0.090%	0.303%

NOTE: Test results through the HPLC/MS methods listed here.

It can easily be seen from this table that there is quite a difference in quality of *Cordyceps* from different producers. These quality differences become even more pronounced when seen in graphic format. Plot 1 below is shows the HPLC analytical profile of the hybrid *Cordyceps sinensis* strain overlaid on a plot of wild *Cordyceps sinensis* to show the comparison in qualitative similarities as well as the quantity of active ingredients in the cultivated hybrid strain verses the wild strain of *Cordyceps*. The secondary metabolites produced are very nearly identical in these two specimens of *Cordyceps* as reveled by these overlaid analytical signature.

PLOT 1 – WILD *CORDYCEPS* AND HYBRID *CORDYCEPS*



TOTAL HEAA CONTENT IN THIS SAMPLE OF WILD CORDYCEPS IS ABOUT .091% w/w

THE HEAA CONTENT OF THE HYBRID CORDYCEPS IS .323% W/W

NOTE: This plot of the wild *Cordyceps* is the basic signature of *Cordyceps sinensis* that we use for comparative purposes when determining whether a sample is actually *Cordyceps* or not. An unknown sample is considered as "authentic" *Cordyceps sinensis* if the plot of the unknown sample matches most points (>90%) on this natural *Cordyceps* plot when one is overlaid on the other.

We further showed through HPLC and GC analysis that *Cordyceps* is by no means consistent in its profile of secondary metabolites, but rather the analytical profile varies greatly, according to the combination of all the differing parameters laid out in this article. While different strains seem to have the greatest influence in exactly which secondary metabolites are produced, all factors involved in the entire cultivation process come into play in this regard. Even subtle changes in light cycle can drastically influence the resulting chemical profile.

DISCUSSION

We found great differences in final cultivated *Cordyceps* product quality, dependent on several factors, such as strain, substrate and culture parameters followed. The Milo or Milo and Millet based substrates proved to be the best substrates of all that we tested, based on the quantities of the secondary metabolites we were measuring (HEAA). If other end products are desired, then perhaps other substrate compositions will prove more valuable. The only way to determine the end result is to try different substrates and analyze the resulting mycelium. Parameters that are commonly used in farming edible mushrooms such as speed of growth, density, or color were found to be generally unreliable indicators of *Cordyceps* quality, if quality is to be defined in terms of concentration of bioactive compounds. These "target compounds desired" should be

used as the basis for formulating substrate composition, with composition used as one of the three arms of strategic husbandry to insure the medicinal properties are optimized. The other two arms of optimum cultivation, culture parameters and strain, are no less important, and all three factors must be evaluated together.

The HPLC analytical protocols presented in this paper, and to a lesser degree the GC protocols, allow quick and relatively inexpensive methods of analysis to guide the cultivator in their search for quality. Until better protocols are developed or further bioactive compounds are identified, we urge all cultivators of medicinal *Cordyceps* to adopt these test methods, so that all the different *Cordyceps* on the market will be evaluated consistently. By taking the approach of adding together the quantities of several different compounds, the total 'Quality Index' of dissimilar products can equate, while specific levels of this or that compound still vary due to strain or growing conditions. Further, if and when customers call for greater quantities of any specific compound(s) in their product, these test methods offer rapid means of developing new cultivation strategies, again based around the target compounds desired.

These same comments of repeated trial and analysis apply equally to the specific cultivation parameters, such as high temperature for the production of higher quantities of Adenosine or low temperature hypoxia for production of greater quantities of the deoxygenated nucleosides. This work in modifying the culture parameters suggests that other parameters may yield some interesting compounds, such as high sulfur content in the substrate or atmosphere for the production of sulfated polysaccharides, such as ones that have shown promise as antifungal and antiviral agents. (Wasser and Weis, 1999)

It is proposed by these authors that the simple addition of components to the substrate, coupled with carefully controlled growing conditions could result in the production of some wonderfully unique compounds by this fungus. We have even grown *Cordyceps sinensis* on a substrate consisting primarily of chocolate milk, and one composed solely of ground squid. These substrates resulted in some very unique compounds produced, the usefulness of which have yet to be determined.

Hybridization of fungal strains is a relatively new practice, and the simple low-cost methods shown here allow the average cultivator a way to develop their own strains, suited to their particular requirements. Perhaps the cultivator has access only to some substrate material on which the *Cordyceps* grows but poorly. Here then is a method that can be applied to "create" a new strain, which will fulfill the intended function, growing on this substandard medium. This is only one of nearly limitless uses for this hybridization technique. We have found that in some cases, we are even able to hybridize between different species, such as crosses between *Cordyceps sinensis* and *Cordyceps sobolifera*. This may indicate that these two species are closer together in the evolutionary tree than was previously thought, or it could mean that we have a method by which to trick nature into performing that which does not take place naturally. But whatever the explanation, it certainly allows us to create unique new strains with which to work.

CONCLUSION:

There is a large and growing market worldwide for *Cordyceps* as a Medicine and as a Health Supplement. This large market demand coupled with the high prices of wild *Cordyceps* has

given rise to an ever-increasing cultivation attempt by mushroom cultivators in many countries. Until now, there has been no standard of quality, and no well-understood cultivation protocols with which to consistently produce high quality *Cordyceps*. Compounding this has been the lack of any standardized test method by which to analyze and ascertain whether the cultivated *Cordyceps* was of a good quality or not. Here we have shown how hybridization results in higher quality *Cordyceps*, and have presented an overview of cultivation techniques, bioactive target compounds and quality index references, analytical protocols, substrate composition, cultivation methodology and hybridization techniques for production of the cultivars necessary in accomplishing these ends. This should serve as an outline for the production of high quality, economical *Cordyceps*, suitable for pharmaceutical and nutraceutical use, at a consistently high level of bioactive ingredients. This is a safe, natural and traditional medicine with a long history of use that is now available and ready to be used on an ever-increasing scale in the treatment of modern diseases.

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